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PharmTech
International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.2, No.1, pp 644-648, Jan-Mar 2010

In vitro antibacterial activity of bioactive metabolite and crude extract from a new *Streptomyces* sp. *Streptomyces rajshahiensis*

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Abstract: A new strain of *Streptomyces* identified as *Streptomyces rajshahiensis* (RUPA08-PR) was isolated from the soil of Rajshahi, Bangladesh. Ethyl acetate was the most convenient solvent for the extraction of antimicrobial metabolites from the culture filtrate of this strain. From the ethyl acetate extract compound **1** was isolated, purified and characterized by spectral data. The compound showed antibacterial activity against a number of both Gram positive and Gram negative bacteria but did not have antifungal activity. The minimum inhibitory concentration (MIC) of the compound **1** and ethyl acetate extract was determined against four Gram positive and four Gram negative bacteria and the values were found between 32 and 128 µg/ml. Both the isolated compound **1** and the ethyl acetate extract exhibited cytotoxic effect in brine shrimp lethality bio-assay with LC_{50} values of 1.32µg/ml and 0.89µg/ml respectively.
Key words: *Streptomyces rajshahiensis*, antimicrobial activity, brine shrimp lethality bio-assay, cytotoxicity

Introduction

The genus *Streptomyces* is represented in nature by the largest number of species and varieties, producing the majority of known antibiotics among the family Actinomycetaceae. Bacteria belonging to the genus *Streptomyces* are widely recognized as sources of antibiotics and other important novel metabolites, including antifungal agents[1], antitumor agents[2], antihelminthic agents[3] and herbicides[4] and are used not only in the treatment of various human and animal diseases but also in agriculture and biochemistry as metabolic poisons.[4-6] At least 70 of the approximately 100 marketed antibiotics used for the treatment of infections in humans are derived from substances produced by *Streptomyces* sp. for example *Streptomyces aureofaciens* is an important industrial microorganism as a producer of chlortetracycline and tetracycline[8]. It is generally accepted that the 'golden age' of antibiotic discovery from actinomycetes has passed, since fewer antibiotics are being discovered now than in the 1950s and1960s, nevertheless these microorganisms remain a rich source of novel bioactive compounds; one study estimates that the number of antibiotics produced by the genus *Streptomyces* is of the order of 100,000, thus many more antibiotics remain to be identified.[9] Discovery of new antibiotics produced by streptomycetes still continues, such as actinomycins X_2 produced by *S. nasri*[10] and pyrroindomycins produced by *S. rugosporus*.[11]

In our continued search for novel microbial metabolites[12-13] having agricultural and pharmaceutical potential, a number of *Streptomyces* strains were screened.[14] This has resulted in isolation of a promising new strain of *Streptomyces* species[14] from a soil sample collected in the region of Rajshahi. We,

herein, report the isolation and identification of compound **1** from this new species. Antimicrobial, minimum inhibitory concentration and cytotoxic activities of the compound and crude ethyl acetate extract against some pathogenic bacteria and fungi are also described.

Materials and methods

Collection and identification of organism

The organism was isolated from a soil sample collected from Rajshahi, Bangladesh at the depth of 0.75m using crowded plate technique.[15] The organism was identified as a novel *Streptomyces* species on the basis of morphological, physiological, biochemical[16] and 16S rDNA studies (GenBank accession number BankIt1256035 GQ500975) and designated as *Streptomyces rajshahiensis* (RUPA-08PR).[14] Pure culture of the strain was maintained on Czapek Dox (alkaline) agar slant.

Fermentation

The strain was grown on modified Czapek Dox (alkaline) agar slant at 37.5°C for 5 days. Spores were collected from a slant culture with 10 ml of the same medium broth. Cultivation of the strain was made by transferring 1ml (ca. 10^8 cells/ml) of the spore suspension and was incubated at 39°C (at 250 rpm) for ten days in 500 ml Erlenmeyer flasks containing 100 ml of antibiotic medium consisted of 3 % glucose, 0.2 % yeast extract, medium containing KCl (0.05%), KH_2PO_4 (0.1%), $MgSO_4$, $7H_2O$ (0.05%) and $FeSO_4$, $7H_2O$ (0.001%). and 1% NaCl.

Extraction, isolation and purification of compound

The maximum production of antimicrobial metabolites from the strain was obtained at the 10th day of incubation in modified Czapek Dox broth (alkaline) medium at 39°C by maintaining all other physicochemical factors in optimum conditions for the culture.[14] The extraction of the bioactive metabolites was carried out by ethyl acetate on the basis of best solubility and maximum antimicrobial activities. The culture broth (50 × 200 ml) of *S. rajshahiensis* was partitioned with ethyl acetate (50 × 60 ml) and concentrated to dryness by using a rotary evaporator under vacuum at 40°C. The extract (8 gm) was subjected to column chromatography on column-graded silica gel with gradient elusion using n-hexane and ethyl acetate mixtures. From the fraction FAR-01 (1.3 gm), a compound was purified on preparative-TLC (applied on silica gel 60, PF254+366 MERCK; glass plates 20 mm and 20mm, 0.25 and 0.5 mm MERCK) using n-hexane: ethyl acetate (3:1) as eluent with the help of UV light (254 and 366 nm) and vanillin- sulfuric acid spray reagent followed by heating at 120°C and yielded compound **1** (22 mg, the compounds were light brown oily mass with characteristic odor, mp. 115-125 °C).

Antibacterial and antifungal screening

Both antibacterial and antifungal activities of compound **1** and ethyl acetate extract were observed by disc diffusion assay.[17-18]. A total of five Gram positive and nine Gram negative bacteria were used in this antimicrobial screening. Compound **1** and ethyl acetate extract (150µg/disc and 30µg/disc) were prepared by dissolving with ethyl acetate. To compare the antibacterial activity, kanamycin (30µg/disc) was used as standard antibiotic. As a negative control, a blank disc impregnated with solvent followed by drying off was used. The antifungal activity of the compound **1** and ethyl acetate extract were tested against five pathogenic fungi at a concentration of 150 µg/disc. Potato dextrose agar (PDA) media used for this purpose. The activity was determined after 72 hours of incubation at room temperature (37 ºC). Nystatin was used as standard at a concentration of 30 µg/disc.

Minimum inhibitory concentration (MIC) assay

The minimum inhibitory concentrations (MIC) were determined by serial dilution technique[19] in the presence of standard Kanamycin. Dimethyl sulphoxide was used for dilutions of the coordination complexes under test in MIC determination. Bacterial inocula was prepared at 5×10^6 - 5×10^7 cfu/ml. Final adjustment were made using optical density measurement for bacteria (absorbance 0.05 at a wavelength of 660 nm).

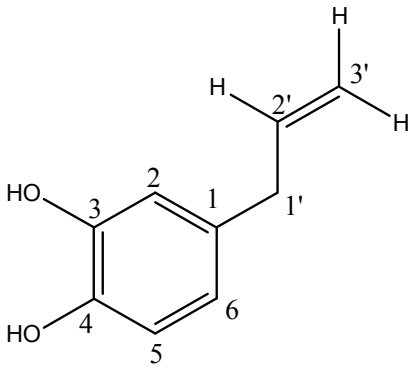
Cytotoxicity screening

For cytotoxicity screening, DMSO solutions of the compounds were applied against *Artemia salina* for 24 hrs *in vivo* assay.[20] The eggs of the brine shrimp, *Artemia salina*, were collected from a local aquarium shop and hatched for 48 hr to mature shrimp called nauplii.20 The test samples were prepared by dissolving them in DMSO (not more 3.8% NaCl in water) to attain concentrations - 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml. A vial containing 50 µl DMSO diluted to 5ml was used as a control. Then 20 matured shrimps were applied to each of all experimental vials and control vial. The number of the nauplii that died after 24 hr was counted. The findings were presented graphically by plotting log of concentration versus percentage of mortality of nauplii from which LC_{50} was determined by extrapolation. The assay was performed in duplicate and the result was calculated as an average of two determinations.

Results and discussion

Characterization by spectroscopic method

Column chromatography followed by preparative TLC on the ethyl acetate extract of *S.* rajshahiensis yielded compound **1**.



The ^{1}H NMR experiment (500 MHz) of **1** showed ABX pattern aromatic protons at 7.55 (1H, *d*, *J* = 8.5 Hz), 7.14 (1H, *dd*, *J*= 8.5, 2.5 Hz) and 7.37 (1H, d, *J*= 2.0 Hz). In the COSY experiment these protons showed expected coupling among themselves. In the HMBC experiment, the methine proton at 7.55 was connected to two quaternary carbons at 147.8 (C-3) and 131.1 (C-1) while the other two methine protons at 7.14 and 7.37 showed ^3J correlations to oxygen bearing quaternary carbon at 148.8 (C-4).

The signals at δ 5.01 (1H, d, *J*= 17.5 Hz), 4.94 (1H, d, *J*= 11.0 Hz), 5.82 (1H, m), and 2.05 (2H, m) in the ^{1}H NMR spectrum were indicative of a prop-2-enyl side chain in the molecule (Table 1). In the HMBC experiment, the methylenes at 2.05 showed correlations to methine (139.5) and methylene (114.3). Based on above correlations the compound 1 was identified as 1-prop-2-enyl-3,4-dihydroxybenzene.

The results of antibacterial activity are presented in Table 2. The fractions and pure compound **1** showed reasonable antibacterial activity against pathogenic bacteria. However, compound **1** was more active against Gram positive than Gram negative bacteria. The antifungal activity of compound **1** was very low compared to standard but crude extract showed moderate activities (Table 3). The minimum inhibitory concentrations (MICs), determined by serial dilution technique[19] of compound **1** were shown in Table 4. The MIC values of compound **1** were between 32-64 µg/ml and for crude extract 16-32 µg/ml respectively. Further work is necessary in order to establish the antibiotic importance of the isolated compound. The cytotoxic activities of the compound **1** and ethyl acetate extract were studied by brine shrimp bioassay. The LC_{50} value of the compound **1** and ethyl acetate extract were found 1.32µg/ml and 0.89µg/ml, respectively.

Table 1: ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and HMBC (500 MHz) data of compound 1 in $CDCl_3$

Position	^{1}H	^{13}C	HMBC (H→C)
1	-	131.1	
2	7.37, 1H, *t*, *J*=2.0 Hz	124.6	
3	-	147.8	148.8 (C-O), 124.2 (CH)
4	-	148.8	
5	7.55, 1H, *d*, *J*= 8.5 Hz	119.9	148.8 (C-O), 124.6 (CH)
6	7.14, 1H, *dd*, *J*= 8.5, 2.5 Hz	124.2	147.8 (C-O), 131.1 (C)
1’	2.05, 2H, *m*	32.2	114.3 (CH_2), 139.5 (CH)
2’	5.82, 1H, *m*	139.5	-
3’	5.01, 1H, *br d*, *J*=17.5 Hz	114.3	32.4 (CH_2)
	4.94, 1H, *br d*, *J*=11.0 Hz		

Table 2. Antibacterial activity of crude extract (ethyl acetate) and compound 1 of *S. rajshahiensis*

Test organism	Diameter of the zone of inhibition (mm)		
	EA 150 µg/disc	Compound 1 150 µg/disc	Kanamycin 30 µg/disc
Gram positive			
Bacillus subtilis QL-40	22	16	27
B. megaterium QL-38	20	14	28
Sarcina lutea QL-166	17	14	31
Staphylococcus aureus ATCC-259233	16	15	34
Streptococcus-β-haemolyticus CRL	18	17	33
Gram negative			
Escherichia coli FPFC-1407	20	12	28
Psedomonas aeruginosa CRL	16	10	33
Salmonella typhi	17	19	29
Shigella boydii AL-17313	16	18	32
Sh. dysenteriae	17	17	29
Sh. sonnei AJ-8992	19	19	34
Shigella flexneri AL-30372	18	16	27
Klebsiella species	10	12	28

EA = Ethyl acetate ;

Table 3. Antifungal activities of crude extract (ethyl acetate) and compound 1 of *S. rajshahiensis*

Test organism	Diameter of the zone of inhibition (mm)		
	EA 150 µg/disc	Compound 1 150 µg/disc	Nystatin 30 µg/disc
Candida albicans	15	-	23
Aspergillus fumigatus	12	7	21
Aspergillus niger	18	6	25
Aspergillus flavusr	16	7	27
Epidermophyton floccosum	14	-	28

Table 4: The MIC values of the ethyl acetate extract and compound 1 of *S. rajshahiensis*

Test organism	EA MIC (µg/ml)	Compound 1 MIC (µg/ml)
Gram positive		
Bacillus subtilis	16	32
Sarcina lutae	32	64
Staphylococcus agalactiae	32	32
Staphylococcu aureus	16	32
Gram negative		
Shigella dysenteriae	32	64
Escherichia coli	32	64
Shigella shiga	64	64
Pseudomonas aeruginosa	32	64

Acknowledgement
The authors wish to thank Institute of Nutrition and Food Science (INFS), University of Dhaka and International Center for Diarrhea Disease and Research of Bangladesh (ICDDRB) Dhaka for providing pathogenic microorganisms for the study.

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